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(54) Title: PROTECTION AGAINST PATHOGENIC MICROORGANISMS

(57) Abstract

The present invention relates to a bacteriostatic and bactericidal Carnobacterium. In particular one having the accession number DSM 10087 as deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and/or an inhibitory compound produced by said strain.

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Protection Against Pathogenic Microorganisms

The present invention relates to a nutritionally and prophylactically valuable product to improve the gut microbiological flora in mammals, including man, and in fish, shellfish and mollusces.

It is recognised that the status of the microbiological flora in the gut of an animal may have a profound effect on the wellbeing of the animal. Poor status of the gut microflora may result in less than optimal utilization of food and poor growth rate, in lower production of the lower quality of products such as milk, eggs, hide and carcass and/or in greater susceptibility of the animal to disease which may be of longer duration or of greater severity when the gut microflora is poor.

There has therefore been considerable interest in studying the gut microflora of animals, in ways of establishing and maintaining a beneficial microflora and in the mechanisms behind the observed benefits conferred by a gut microflora of the correct status. These investigations have concentrated on humans and on livestock of commercial significance. Thus the farmed

species that have been studied most are those such as chickens and pigs, which commonly suffer from intestinal diseases that may be treated or prevented by intervention aimed at improving or modifying the gut microflora. Even in the absence of disease, commercially significant improvements in performance may be achieved by manipulating, or changing, the gut microflora.

The type of intervention to alter the gut microflora may take several forms. Treatment with antibiotics is often practised to eliminate pathogenic microbes from the gut. This is usually accompanied by a reduction of those naturally resident microbes considered neutral or beneficial. Another form of intervention is to ensure that food contains constituents that promote the growth of beneficial microbes. Yet anther form of intervention is to deliberately treat the animals with a live population of beneficial microbes, usually by including such microbes in the food or drinking water.

Treatment with live microbes is increasingly practised. The objective is to ensure that an adequate microflora of the desired microbes is established in the gut at an early age of the animal or introduce into a potentially disrupted gut ecosystem. The microflora becomes established within the gut by association with the gut mucosa and by colonizing the lumenal contents. Some microbes may adhere directly to the mucosal epithelium while others may be resident within the mucilage that lines the gut. The interactions between the microbes and the host are complex but some detailed understanding of microbe-microbe interactions and of the surface interactions between gut microbes and mucosal cells is emerging.

The detailed mode of action of the gut microflora is not well understood, either with respect to the biochemical reactions mediated by the whole population or with respect to the activities of the individual microbes within the population. This is particularly true when considering effects on digestion and uptake of nutrients. There is, however, more information concerning the effects of gut microflora organisms on health aspects. Such organisms have been studied with respect to their activation of dietary, especially xenobiotic, compounds to carcinogens, and detoxification activity by desirable microbes: the potentiation of both non-specific and specific immunological defence mechanisms by desirable microbes: and the antagonism against pathogenic microbes by desirable microbes.

The effects of gut microbes on enteric pathogens such as Salmonella, Clostridium, and E. coli have been studied. Beneficial microbes can suppress or prevent the colonization of the intestine by pathogens. The mechanisms known or inferred are varied. There may be very specific mechanisms such as the production of a specific antimicrobial substance, such as bateriocin, by the beneficial microbes(s). There may be a production of broad range antimicrobial such as reuterin active against many bacteria, yeast and fungi. Other chemical inhibitors of pathogens produced by beneficial microbes include organic acids (in particular lactic acid) and hydrogen peroxide. Pathogens may also be selected against by the physical conditions of pH and redox potential controlled by beneficial microbes in the gut. Beneficial microbes may also prevent pathogens from becoming established in the gut by superior competition for nutrients or by occupying sites on the gut mucosa or within the mucus

overlying the epithelial cells that would be required by the pathogens for their colonization. A number of these actions are encompassed within the term "competitive exclusion".

Background to the present invention

In the studies of adjustment of the gut microflora in humans and animals such as chickens, pigs and other effective treatments have been shown to consist of administration of live cultures of microbes which consist of or include bacteria belonging to the group described as "lactic acid bacteria". Such cultures are often termed "probiotic" preparations. The administration method is usually to provide the culture in the food or drinking water although spraying of newly hatched chicks and their surroundings has also been effective. Successful administration may be monitored by assessing the establishment of the supplied microbe(s) in the animal's digestive tract.

The use of lactic acid bacteria as probiotic microbes stemmed from the early work of Metchnikoff with human infants and this group of bacteria has featured in much of the later work in both humans and animals. It appears that many mammals do have lactic acid bacteria as beneficial microbes in their digestive tract but other microbes, such as Bacillus, and yeast and fungi can be effective. In avian species, lactic acid bacteria are also important in probiotics but obligate anaerobic bacteria are also claimed to be necessary for protection against salmonellosis. In other cases a mixed culture of relatively few (less than 10) types of related microbes may be necessary; in yet other cases a complex mixed culture containing many tens (perhaps 100) of different types of microbes may be effective.

The culture used in this last case may approximately the entire resident beneficial microflora in the gut of the target animal.

The source of probiotic microbes can be food such as fermented milk products, like yoghurt, in the case that it is desired to established or improve the population of particular lactic acid bacteria in the gut, or deliberately isolated cultures from the intestines of the target animal. In the latter instance, successful probiotic samples have been isolated from faecal samples but the microflora of faeces represents largely the transient microbial population in the gut whereas it is often desired or advantageous to employ a culture that is representative of the resident microbial microflora in the gut in order to ensure that isolation of the potential probiotic strain takes place from the resident microflora, it is necessary to carry out isolations from the alimentary canal directly, often from a location where it is desired to encourage the probiotic strain to reside. Thus scrapings of the internal intestinal mucosa of recently sacrificed, healthy animals may be a source for isolation of suitable organisms for testing as probiotics.

It is observed that the distribution of the gut microflora in both qualitative and quantitative aspects is not uniform along the length of the alimentary canal. Frequently, the greatest and most varied populations are found in the lower intestine. It is here that the gut microflora probably exerts its major effect on digestion, nutrient uptake and also intestinal colonization by pathogens.

After a population of the resident gut microflora has been isolated, it may be used as such to inoculate an

animal with an inadequate gut microflora or it may be resolved into individual microbial strains for reintroduction as single strains or as simplified mixed populations. In the case that it is desired or necessary to separate a mixed population into its individual microbial strains, the techniques commonly employed in microbiology may be used. In particular separations may be made based upon the morphology of colonies on various solid and liquid media grown with different carbon and energy sources, with different nitrogen sources, under different conditions of gas supply (aerobic and anaerobic), at different pH values and other conditions known to those skilled in the art.

If individual microbial stains are to be selected from a mixed population for use as probiotic strains it is necessary to apply some practical criteria for their selection. These criteria are partly dependent on the required attributes of a probiotic strain and include:

- origination from the animal species in question;
- sufficient stability to digestive conditions (acid, bile, enzymes) to allow survival;
- ability to colonize the animal species in question under practical conditions. This may include the ability to adhere to the intestinal cells although effective strains may be able to reside within the intestinal mucus or lumenal contents without direct contact with the intestinal mucosal cells;
- antagonism against potential microbial pathogens. This may include the production of general or specific antimicrobial substances by the selected strain.
- safety in use. This includes the demonstration that the selected strain is not itself a pathogen causing a clinical disease.

It will be apparent that individual microbial strains isolated from a population originating from resident gut microflora of a healthy animal should meet many of these criteria by definition: including origination from the species in question and sufficient stability and ability to colonize the animal gut. In terms of selective criteria for choosing a probiotic strain, the ability to adhere to intestinal mucosal cells may be applied in the case where it is known that the microbe must adhere to such cells, for example, when the strain is to be applied to new born or newly hatched infants which have a naked, or nearly so, gut mucosa. The inability of a strain to adhere to the gut mucosa, does not, however, indicate that the strain is without utility as a probiotic.

In the case that one objective of using a probiotic is to combat pathogenesis via the gut, selection based on demonstrated antagonism towards likely or actual pathogens in the gut of the species concerned is indicated. Numerous in vitro methods of showing and quantifying such antagonism are known to those skilled in the art of selecting microbes producing antibiotics and may be applied here, is recognized, however, that antagonism, or the extend of antagonism, may vary depending on the in vitro methods used. It is also recognized that it may be difficult to show in vivo the same antagonism which can be demonstrated in vitro, partly because it is difficult to reproduce exactly the in vivo conditions in the laboratory experiments. However, the effectiveness of the probiotic may be readily demonstrated by subjecting the animal treated with the probiotic strain to challenge with a disease causing microbe.

Since the gut microflora may harbour pathogenic

microbes in the carrier state and hence there are no signs of clinical disease, it is clearly necessary to exclude such pathogens from the selected population of probiotic strains. Suitable tests to establish the non-pathogenicity of a test probiotic strain include deliberate injection into the animal. One route for injection in such tests is into the peritoneal cavity. Observation of lack of disease symptoms and inability to isolate live microbes of the test strain from the target organs indicates lack of pathogenicity.

It is known that fish harbour bacteria with inhibiting activity against pathogens in their gut microbial flora. Thus Westerdahl, A., et al, Appl Environm. Microbiol. 572223-2228 (1991), and Olsson. J. C., et al, Appl En-vironm. Microbiol. 58:551-5556 (1992) discloses isolation and characterization of turbot associated bacteria with inhibitory effects against Vibrio anguillarum.

US-A-4,657,762 discloses a composition useful in the treatment of disturbances in the normal intestinal flora of poultry, whereby the composition contains anaerobic bacteria of intestinal origin.

Austin B., et al, J. Fish Disease, 15:55-61 (1992) discloses inhibition of bacterial fish pathogens by Tesraselmis suecica by administering supernatants and extracts from heterotrophically grown Tetraselmis suecica which inhibit different prawn pathogenic vibrios.

Robertson, B., et al, J Fish Diseases 13:291-400 (1990) discusses enhancement of non-specific disease resistance in Atlantic salmon, Salmo salar L., by a glucan from Saccharomyces cerevisiae cell walls when

injected intraperitoneally.

Smith, P., et al, J. Fish Diseases. 16:521-524 (1993) discloses evidence for the competitive exclusion of Aeromonas salmonicida from fish with stress-inducible furunculosis by a fluoroscent pseudomonad isolated from gill and gill mucus of brown salmon, Slamo trutta L, which strain had been isolated for its ability to inhibit Aeromonas salmonicida.

Douillet, P, A., et al, Aquaculture, 119:25-40 (1994) discloses the use of probiotic for the cultures of larvae of the Pacific oyster (Crassostrea gigas Thunberg) whereby addition of strain CA2 as a food supplement to xenic larval cultures of the oyster Crassostrea gigas enhanced growth of the larvae.

Description of Present Invention

It has now surprisingly been shown that a bacterium found within a bacterial population isolated from one Atlantic salmon, Salmo salar, exhibits strong inhibitory effects against bacterial fish pathogens, Vibrio anguillarum, (vibriosis), Vibrio ordalli (vibriosis), Aeromonas salmonicida, (furunculosis), and others. The strain which has been denoted strain K in the following has been provided the accession number DSM 10087 as deposited with the Deutsche Sammiung von Mikroorganismen und Zellkulturen GmbH on 6 July 1995 under the Budapest Treaty.

The strain K, as hitherto isolated from an Atlantic salmon, in accordance with Olsson, J. C., et al, Appl Environm. Microbiol. 58:551-556 (1992) (enclosed herein as a reference) proved to be a motiel, Gram-positive pleomorphic, facultative anaerobic rod. The

antibacterial activity of the strain K was analysed and the results suggested that multiple, broad range antibacterial compounds are released in the surrounding medium during the logarithmic phase of growth in TSBS (Tryptic Soya Broth supplemented with Salt, NaCl 2% w/v) medium. The inhibitory compounds were also produced when the strain K was grown in diluted intestinal mucus, which suggests that the strain K bacterium will proliferate and produce the antibacterial substance in the gut. The antibacterial compounds were heat labile, and were initially determined to have a molecular weight of about 140-150 dalton by gel filtration. The antibacterial compounds have been found to have an inhibitory activity against both Gram-negative and Gram-positive bacteria, but not against yeast. The antibacterial compounds are bacteriostatic at low concentrations but bactericidal at higher concentrations. The activity is maintained when the compounds are stored in frozen state, but is lost when maintained at 23°C.

Strain K shows the following phenotypic characterization:

The major fatty acids are 16:0 (31.1%); 16:1 (24.2%); and 18:1 cis 9 (23.4%). The remaining fatty acids are: 18:2 cis 9, 12; 18:0A (10.8%); 14:0 (5.4%) and 18:0 (3.5%). Strain K can utilize the following carbon sources: sucrose, maltose, trehhalose, mannitol, ribose, B-D-glucopyranoside. It was not able to produce acids from the following carbon sources: cyclo-dextrin, tagotose, D-arabitol, L-arabinose, melezitose, melibiose, pullulan, glycogen, raffinose, lactose, and sorbitol. It did not produce acetoin. It was able to hydrolyse hippurate. Beta-Glucosidase activity was demonstrated. No activity of the following enzymes

were detected: urease, betagalactosidase, beta-glucuronidase, alfa-galactosidase, arginin dihydrolase, alanylphenylalanyl-prolin arylamidase, acide pryoglutamique arylamidase, N-acetyl-beta-glycosaminidase.

Strain K is sensitive to gentamycin, erythromycin, rifampicin, tetracyclin, ampicillin, and kanamycin. It is sensitive to a lesser extent to neomycin and nalidixic acid. Strain K does not harbour any detectable plasmids.

A 2.3 fold diluted of a cell-gree culture supernatant provides a total growth inhibition of Vibrio anguillarum (HT11360). Aeromonas salmonicida (ATCC 14174) is more sensitive than the Vibrio anguillarum strain, and a 10 times dilution results in total growth inhibition during a 24 hours test period. Any dilution of the TSBS does not interfere with these results.

The active compounds loses its activity gradually at 4°C and cannot be detected after 8 weeks. When frozen the full activity remains for at least 12 months.

The activite compound of strain K inhibits a large number of bacteria, whereby no difference is seen between Gram-negative bacteria. All pathogens tested were sensitive, whereby Staphylococcus aureas and Proteus vulgaris CCUG 6327 proved to be the most sensitive and E. Coli Av24 and Pseudomonas aeruginosa the least. Yeast is not inhibited.

Strain K grows in intestinal mucus. Growth was proceeded by a lag phase of at least 7.5 hours. This is comparable with the length of the lag phase that is exhibited in TSBS by the same strain in the same

temperature. Strain K was found to produce substances during growth in mucus that are inhibitory to growth of the fish pathogens Vibrio anguillarum and Aeromonas salmonicida. The inhibitors were detected in the mucus at the onset of the logarithmic growth (7.5 hours). An increase in the inhibitory activity was observed throughout the log phase and into stationary phase. The growth of the pathogens was not inhibited in the control culture with intestinal mucus without strain K. The colonies on the TSBS plates were identified as strain K, a Carnobacterium by biochemical tests. The bacteria in the pinpoint colonies were found to be motile, forming pairs or chains with four cells, Gram positive, catalase and oxidase negative. Inhibition zones were around the colonies when tested against V. anguillarum.

The colony forming units (CFU) were found to increase approximately 3 log during 12.5 hours of growth in faeces suspension. No lag phase was seen during growth in faeces suspension. After the culture had reached stationary phase, at 12.5 hours, no further increase of inhibitory activity was detected.

Production of substances that inhibit the growth of the fish pathogens, V. anguillarum and A. salmonicida, was detected after 7.5 hours. The inhibitory activity increased until 12.5 hours and then remained unchanged. V. anguillarum and A. salmonicida grew in the faeces control. The identity of the strain K colonies was confirmed as described in the previous section.

Neither strain K, nor its inhibiting compound (-s), is toxic to fish. No fish in any tested group died or showed any external signs of disease during the experiments in which fish were exposed to strain K.

Spleens from both infected with strain K and control fish were free from culturable bacteria.

Media, diluent and culture conditions

The media and diluent used with Tryptic Soya Broth (TSB, Difco) and TSBS (TSB supplemented with 2% NaC); TSA (TSB + 15% agar) and TSAS (TSA supplemented with 2% NaCl); TSAS soft agar (TSBS + 5% agar); Marine agar (MA, Difco); Nutrient agar (NA, Difco); Brain heart infusion (BHl, Difco); Rogosa (Difco); TCBS Cholera Medium (Oxoid); Marine Minimal Medium (MMM, Neidhardt et al, (1974)); VFI (peptone 1.0g, yeast extract 0.5 g, glucose 0.5g, starch 0.5g; Salmon intestinal buffer (Hickman (1968)); $NaHCO_3$ 1.03g, NaCl 1.97g, KCl 0.16g, $CaCl_2.2H_20$ 2.07g, $MgSO_4.7H_20$ 21.95g, $MgCl_2.6H_20$ 10.67g, agar 15g distilled water 1000ml); VNSS agar (peptone 1.0g, yeast extract 0.5g, glucose 0.5g, starch 0.5g, $FeSO_4.7H_20$ 0.01g, Na_2HPO_4 0.01g, agar 15g in 1000ml NSS); NSS (Nine Salt Solution: NaCl 17.6g, Na_2S0_4 1.47g, $NaHCO_3$ 0.08g, KCl 0.25g, KBr 0.04g, $MgCl_2.6H_20$ 1.87g, $CaCl_2.2H_20$ 0.41g, $SrCl_2.6H_20$ 0.008g, H_3BO_3 0.008g in 1000 ml double distilled water). Horse-blood agar (HBA, TSA supplemented with 5% horse blood.

Bioassay for the detection of inhibitory effect

i) Double-layer agar method.

The plates were screened for inhibition by a modified double-layer agar method described by McLeod and Govnlock (1921). Macrocolonies of the inhibitory bacteria were obtained by spot seeding on agar plates (10 μl of a liquid culture in log phase) and incubating the plates for 18 hours at 23°C. The macro-colonies were treated with

chloroform vapour for 30 min. The pathogen (100µl of a 10 x diluted liquid culture) was then seeded into a tube of melted (temperature to 45°C) TSAS soft agar (3ml) mixed and then poured onto the top of the plates. After incubation for 18 hours the zones of growth inhibition created by the producing colonies were measured as the distance between the edge of the macro colony and the edge of the clearing zone.

ii) Liquid bioassay in microtitre wells

The inhibitory effect was determined as changes in the optical density (OD_{610}), using microtitre spectrophotometer (Bio Tech. Biokinetics). The inhibition assay was performed in microtitre wells (Nunc, 96 wells). The inhibitory bacterium was grown in TSBS at 23°C. The cells were removed by centrifugation and the supernatant was filter sterilized (MFS-25 cellulose acetate filter units, 0.2 µm). The cell free supernatant (150 µl) was transferred to a microtitre well and an equal volume of fresh TSBS (150 µl) was added. Thereafter the target microorganism (3 µl from a logphase culture) was inoculated in the well and the growth was monitored by measuring the optical density at 610 nm for 24 hours. As a control a dilution series with 2% NaCl solution and TSBS was made to relate growth of the pathogen with the amount of medium added. To control for any auto-inhibition by the pathogen, cell-free culture supernatant (from a culture of the pathogens) was treated in the same way as the supernatant derived from the inhibitory strain.

Table 1

The double-layer agar method was used to test growth inhibition of a wide range of bacteria, as well as salmon related yeasts by macro-colonies of isolated strain K.

Organism	GRAM +/-	Inhibition Zone
Strain K	+	-
Vibrio anguillarum HTI 1360	+	+++
V. anguillarum 2129	+	+++
V. anguillarum	+	+++
V. ordalli NCMB 2127	+	++++
V. fisheri	+	++++
Photobacterium angustum S14	-	++++
Aeromonas salmonicida ATCC 14174		++++
A. hydrophila		+++
A. hyrophila NCTC 8049		++++
Escherichia coli B CCUG 214		+++
E. coli Av24		+
Vibrio sp. 4:44		+++
Salmon isolate		+++
Vibrio sp. D2		+++++
Pseudomonas aeruginosa		+
Staphylococcus aureus	+	+++++
Serratia marcescens CCUG 760	-	+++
Micrococcus luteus	+	++++
Proteus vulgaris CCUG 6327	-	+++++
Klebsiella oxytoca CCUG 383	-	+++
Baciluus mageaterium CCUG 1817	+	++++
B. subtilis CCUG 163B	+	++
Acinetobacter calcoaceticus CCUG 12864	-	++++
Streptomyces griseus CCUG 760		++++
Citrobacter freundi CCUG 418	-	++++

Marine yeast Sc18 (unidentified fish isolate) -

Marine yeast Sc3 Saccharomyces cerevisiae CBS 7765 -

Debaryomyces hansenii HFI -

S. cerevisiae CBS 7764 -

Inhibition zone radius (mm): 0 (-); 1-5 (+); 6-10 (++); 11-15 (+++); 16-20 (++++); >20 (+++++)

Table 2

Phenotype of strain K

Property	Strain K
Single rod	+, two polar flagella
Pleomorphic	+
Motility	+
Flagellation	dipolar
Spores	-
Gram reaction	+
Colony diameter	<1 mm
Colony appearance on TSA	Circular; entire; semitranslucent; raised
Pigmentation	Buff
Odour	-
Anaeorbic growth	+
Temperature for growth	4-30°C
pH for growth	5.5 to 9
pH change during growth	7.2 to 6.8
Salinity for growth (NaCl)	0 to 6%
Catalase	-
Oxidase	-
Haemolysis	alfa
Urease	-

Hydrogensulphide production -
Fermentative in Leifson weak acid, no gas

Strain K is thereby identified as a Carnobacterium and was primarily characterized as Carnobacterium alterfunditum. However, further investigations of 16S rRNA demonstrates that Carnobacterium alterifunditum is the closest related organism with a homology of 98.7%. However, according to previous publications (Collins et al, 1987) this would justify describing the isolate as a new species of the genus. Thus the strain K is a new strain, whereby it will be named more specifically later on.

The inhibitory compound(s) was partly purified by fist removing the bacteria cells from a TSBS culture by entrifugatino in the early stationary growth phase (13000 x g for 10 min). The sample was then kept at 4°C during the subsequent purification steps. The cell-free culture supernatant was fractionated by passing it through a 500 dalton cut-off filter (Amicon, Difaflo YC05). Further purification was performed by gelfiltration using a G10 Sephadex (Pharmacia, Sweden) in a XK26/40 column. PBS (2mM) was used as a effluent buffer. The ultra-filtrated supernatant was applied to the coloumn and eluated at a flow rate of 44 ml/h. Fractions (2.9 ml) were collected and examined for antimicrobial activity by the liquid bioassay described above. The apparent size of the inhibiting compound(-s) was determined using a standard curve including NADH (709 D), N-formyl-Met-Leu-Phe-Phe (584.7 D, Sigma), Tyr-Gly-Gly (295.3 D, Sigma), L-tryptophan (204.23 D) and tyrosine (181.19 D) as markers. Blue dextran was used to determine the void volume of the coloumn.

The inhibitorswas extracted using ethyl acetate at pH

2.5 and subsequently purified on TLC using silica gel. The inhibitors are stable for at least 24 hours at a pH of 2 to 11.

The partly purified inhibitory compound(-s) was subjected to heat treatment, various enzyme treatments, metaperiodate treatment, stability tested, and culture media dependency.

The action of the inhibiting compound(-s) was determined as follows. Aeromonas salmonicida from a culture in log phase was inoculated into fresh TSBS to a density of about 1 x 10_6 cells/ml. The culture was incubated for 30 min prior to starting the experiment. To 100 ml culture flasks, 10 ml of the Aeromonas salmonicida culture and a mixture (10 ml final volume) of the partly purified inhibitor supernatant, and NSS (pH 7.2) was transferred such that a series of concentrations of the inhibitor was obtained. The cultures were slowly shaken at 23°C and samples in triplicate were taken to determine the number of colony forming units (CFU) during at time period of 26 hours.

A 4 fold dilution of the cell-free supernatant resulted in total growth inhibition of Vibrio anguillarum (H111360). Aeromonas salmonicida (ATCC 14174) was found to be more sensitive than the Vibrio anguillarum strain and a 10 fold dilution still resulted in a total growth inhibition during the 24 hours test period. The dilution of TSBS did not interfere with the results.

The action of the inhibitory compound(-s) against Aeromonas salmonicida using different dilutions is shown in the Table 3 below.

Table 3

Dilution CFU.ml after

	0	1	2	3	4	5	6	7	8	9	10	11	hrs
2 fold	6.4	1.0	0.2	0.2	0.1	0.05	0.05	0.05	0.0				$x10^9$
4 fold	6.4	4.4	2.0	1.0	0.8	0.2	0.1	0.0					$x10^9$
6 fold	6.4	5.4	3.0	1.9	1.4	1.0	0.8	0.2	0.1	0.05	0.0		$x10^9$

The action of the inhibitory compound(-s) against Vibrio anguillarum as determined as the growth of strain K in TSBS at 23°C as and expressed as the increase in optical density at 610 nm over time will be given below. Further the minimal dilution of free cell culture supernatant causing total inhibition of Vibrio anguillarum. The results are combined in Table 4 below.

Table 4

Incubation Time (hrs)	Increase in Optical density at 610 nm	Minimal dilution arbitrary units
5.5	0.025	30
6	0.05	
6.5	0.08	50
7	0.12	
7.5	0.15	70
8	0.18	
8.5	0.21	80
9	0.23	
9.5	0.25	80
10	0.25	
10.5	0.25	80
16	0.25	80

The present strain K the other strains capable of producing the active compound or chemically related compounds or the active compound derived therefrom and chemically related compounds and derivatives thereof can, in particular, be used in the prophylactic or therapeutic treatment of fish infected by fish pathogens, whereby an amount of the strain K that provides an inoculum allowing the colonization of the fish intestine by the strain K or an amount of the strain K that provides an active amount of the inhibiting compound, is administered to the fish, or an active amount of the inhibiting compound as such is administered to the fish for prophylactic and/or therapeutic treatment of fish susceptible to fish pathogens. Hereby all types of fish are encompassed,

to which strain K is not pathogenic, harmful or deleterious, but in particular salmonids, turbot, yellow tail, seabass/seabream and other types of farmed fish and other aquaculture species, such as shellfish (prawns and shrimps) and mollusces.

It might be so that strain K is pathogenic, harmful or otherwise deleterious as such in some of the organism to which it is administered although this is not foreseen. However, the active compound(-s) therefrom might each be so, but can be administered instead for obtaining a bacteriostatic or bactericidal effect.

The strain K or active inhibiting compound derived therefrom can be administered orally as such or via the feed, which is the best mode, bathing of young or older fish, single inoculation of young or older fish to establish the strain K in the gut, or repeated inoculation of young or older fish. When the active compound as such is administered together with the feed, one has to consider the lability of the compound, if it is to be incorporated into the feed before the hydrothermal forming of pellets. A suitable means to avoid thermal destruction of the active compounds is to add them to the feed pellets after their information and cooling.

The strain K or its active inhibitory compound(-s) can be administered in different ways such as via food, feed-stuff including drinking water, as a composition as such containing the strain. Further it can be added via spraying the animals, including fishes, by immersion of the animals, in particular when fish is concerned, by injection into the gut, or via inhalation.

Claims

1. Use of a probiotic for treating mammals, including man, fish, shellfish and mollusces, comprising at leat one microbial strain isolated from the resident gut microflora of healthy fish and selected by methods known per se to be capable of establishing itself at an effective level in the intestine of the animal treated, whereby the strain is a bacteriostatic and bactericidal Carnobacterium.

2. The use as in Claim 1 of a Carnobacterium having the accession number DSM 10087 as deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

3. The use of said strain of claims 1-2 for the prophylactic treatment of mammals, including man, fish and other aquatic animals.

4. The use of said strain of Claims 1-2 for the therapeutic treatment of mammals, including man, fish and other aquatic animals.

5. The use of the probiotic of Claims 1 to 4, whereby said strain is administered by immersion of the subject in a liquid container said probiotic.

6. The use of the probiotic of Claims 1 to 4, whereby said strain is administered via the food/feed including drinking water, supplied to the subject.

7. The use of the probiotic of Claims 1 to 4, whereby said strain is administered via spraying onto the subject.

8. The use of the probiotic of Claims 1 to 4, whereby the said stain is administered via injection into the subject.

9. The use of the probiotic of Claims 1 to 4, whereby the said strain is administered via inhalation into the subject.

10. The use according to Claims 2 to 9, whereby said strain is used in the treatment of infections caused by Gram-positive and/or Gram-negative bacteria, such as Vibrio anguillarum, Vibrio ordalii, Vibrio fischeri, Aeromonas salmoncida, Photobacterium angustum, Aeromonas hydrophila, Staphylococcus aureus, Bacillus megaterium, Acinetobacter calcoaceticus, Serratia marcescenes, Micrococcus luteus, Proteus vulgaris.

11. A microbe inhibiting active compound derived from strain of Claim 1.

12. The use of a microbe inhibiting active compound of Claim 11 in the prophylactic treatment of mammals, including man, fish, shellfish and mollusces.

13. The use of a microbe inhibiting active compound of Claim 11 in the therapeutic treatment of mammals, including man, fish and mollusces.

14. The use of a microbe inhibiting compound of Claim 12 or 13 for inhibiting the growth of Gram-positive and/or Gram-negative bacteria, such as Vibrio anguillarum, Vibrio ordalii, Vibrio fischeri, Aeromonas salmoncida, Photobacterium angustum, Aeromonas hydrophila, Staphylococcus aureus, Bacillus megaterium, Acinetobacter

calcoaceticus, Serratia marcescens, Micrococcus luteus, Proteus vulgaris.

15. A probiotic for treating mammals, including man, fish, shellfish and molluscs comprising at least one microbial strain isolated from the resident gut microflora of healthy fish and selected by methods known per se to be capable of establishing itself at an effective level in the intestine of the animal treated, whereby the strain is a bacteriostatic and bactericidal Carnobacterium.

16. A probiotic according to Claim 15, wherein the strain is a Carnobacterium having the accession number DSM 10087 as deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in combination with other microbes isolated from a gut microflora of a healthy subject.

17. The use of a bacteriostatic and bactericidal compound expressed by said strain for the treatment of mammals, including man, fish, shellfish and molluscs in combination with other microbes isolated from a gut microflora of a healthy subject.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01936

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/74 C12N1/20 C12P1/04 A23K1/00 //(C12P1/04, C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N C12P A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AQUATIC LIVING RESOURCES, vol. 7, no. 4, 1994, FRANCE, pages 277-282, XP000609995 F.-J. GATESOUPE: "LACTIC ACID BACTERIA INCREASE THE RESISTANCE OF TURBOT LARVAE, SCOPHTHALMUS MAXIMUS, AGAINST PATHOGENIC VIBRIO." see page 281 --- -/--	1-10,15

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 1996	06. 12. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Ryckebosch, A

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/01936

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	APPLIED ENVIRONMENTAL MICROBIOLOGY, vol. 58, no. 2, February 1992, WASHINGTON, D.C., US, pages 551-556, XP000611262 J.C. OLSSON ET AL.: "INTESTINAL COLONIZATION POTENTIAL OF TURBOT (SCOPHTHALMUS MAXIMUS)- AND DAB (LIMANDA LIMANDA)- ASSOCIATED BACTERIA WITH INHIBITORY EFFECTS AGAINST VIBRIO ANGUILLARUM." cited in the application see the whole document ---	1-10,15
A	CHEMICAL ABSTRACTS, vol. 115, no. 25, 23 December 1991 Columbus, Ohio, US; abstract no. 275401v, A.M. BAYA ET AL.: "BIOCHEMICAL AND SEROLOGICAL CHARACTERIZATION OF CARNOBACTERIUM SPP. ISOLATED FROM FARMED AND NATURAL POPULATIONS OF STRIPED BASS AND CATFISH." page 557; column R; XP002018973 see abstract & APPL. ENVIRON. MICROBIOL., vol. 57, no. 11, 1991, pages 3114-3120, ---	2,16
A	CHEMICAL ABSTRACTS, vol. 115, no. 19, 11 November 1991 Columbus, Ohio, US; abstract no. 202927m, P.D. FRANZMANN ET AL.: "PSYCHROTROPHIC, LACTIC ACID-PRODUCING BACTERIA FROM ANOXIC WATERS IN ACE LAKE, ANTARCTICA; CARNOBACTERIUM FUNDITUM SP. NOV. AND CARNOBACTERIUM ALTERFUNDITUM SP. NOV." page 542; column L; XP002018974 see abstract & ARCH. MICROBIOL., vol. 156, no. 4, 1991, pages 255-262, ---	2,16

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INTERNATIONAL SEARCH REPORT

Interr ual Application No
PCT, GB 96/01936

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 117, no. 7, 17 August 1992 Columbus, Ohio, US; abstract no. 66155v, G. STOFFELS ET AL.: "PURIFICATION AND CHARACTERIZATION OF A NEW BACTERIOCIN ISOLATED FROM A CARNOBACTERIUM SP." page 429; column R; XP002018975 see abstract & APPL. ENVIRONM. MICROBIOL., vol. 58, no. 5, 1992, pages 1417-1422, -----	11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 96/01936

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-10, 12-14, 17
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.